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ORIGINAL RESEARCH

# Viridans Streptococcal Biofilm Evades Immune Detection and Contributes to Inflammation and Rupture of Atherosclerotic Plaques

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**BACKGROUND:** Bacterial DNA from the oral cavity, respiratory tract, gut, and skin has been detected in atherosclerotic plaques, suggesting a role in chronic inflammation linked to atherosclerosis. Chronic bacterial infections often form biofilms resistant to antibiotics and immune detection, giving rise to a new generation of virulent bacteria in suitable conditions. This study explores the role of the immune system in bacterial-induced inflammation of atherosclerotic plaques.

**METHODS:** Coronary plaques from 121 sudden death victims and endarterectomy samples from 96 surgical patients were analyzed using bacterial real-time quantitative polymerase chain reaction, immunohistochemistry, and genome-wide expression analysis. TLR (toll-like receptor) signaling was examined in bacterial-activated TLR cell lines.

**RESULTS:** Of the bacteria detected, oral viridans group streptococcal DNA was the most common, being found in 42.1% of coronary plaques and 42.9% of endarterectomies. Immunopositivity for viridans streptococci correlated with severe atherosclerosis ($P<0.0001$) in both series and death from coronary heart disease ($P=0.021$) or myocardial infarction ($P=0.042$). Viridans streptococci colonized the core of the atheroma as a biofilm unrecognized by macrophages of the innate immune system. In contrast, immunopositive streptococci that appeared to have originated from the biofilm infiltrated the ruptured fibrous cap of the atheroma in endarterectomy samples and coronary plaques and were detected by pattern-recognizing receptors and coexpressed with the adaptive immune response. Among the viridans streptococcal strains, TLR2 was the most activated bacterial-signaling pathway. Genome-wide expression analysis of endarterectomy samples showed upregulation of bacterial recognition pathways.

**CONCLUSIONS:** Latent chronic bacterial inflammation evades immune detection and may contribute to the pathogenesis of complicated atherosclerotic plaques and fatal myocardial infarction.

**Key Words:** bacteria ■ biofilm ■ coronary heart disease ■ immune system ■ myocardial infarction

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## RESEARCH PERSPECTIVE

### What Is New?

- Coronary plaques and clinical endarterectomy samples harbored DNA from several oral bacteria among which oral viridans streptococci were the most common, being found in phagocytes and as a biofilm inside atherosclerotic plaques as examined by bacterial immunohistochemistry.
- Viridans streptococcal biofilm was not detected by macrophages of the innate immune system, which, in contrast, detected virulent streptococci that were dispersed from the biofilm infiltrating the ruptured fibrous cap of the atheroma of people experiencing sudden cardiac death, also causing activation of the adaptive immune system in support of the infection hypothesis of myocardial infarction.

### What Question Should Be Addressed Next?

- Could a short-term antibiotics treatment given at the acute phase affect the outcome of myocardial infarction, and would it be possible to develop new diagnostic imaging and prevention methods for bacterial biofilm?

### Nonstandard Abbreviations and Acronyms

| | |
|---|---|
| **ATCC** | American Type Culture Collection |
| **GWE** | genome-wide expression analysis |
| **MyD88** | myeloid differentiation primary response protein |
| **NF-kB** | nuclear factor kappa-light-chain-enhancer of activated B cells |
| **TLR** | toll-like receptor |
| **TSDS** | Tampere Sudden Death Study |
| **TVS** | Tampere Vascular Study |

The present view on the pathogenesis of atherosclerosis entails that it is a chronic subclinical inflammation driven by oxidized low-density lipoprotein (oxLDL) cholesterol. Inflammation is key in both the initial development of the lipid-containing atheroma and its transformation into a vulnerable rupture-prone coronary atheroma.[1–4] Experimental evidence suggests that an important step in the inflammation inside a coronary atheroma is the generation of immune response against oxLDL and its main epitope, phosphocholine.[5,6] OxLDL is recognized by the innate immune system, using germline-encoded pattern recognition receptors, including TLRs (toll-like receptors).[7] Interestingly, although being the major epitope of oxLDL, phosphocholine is also found on a variety of bacteria, such as *Streptococcus pneumoniae*, in which phosphocholine is a prominent constituent of the lipoteichoic acid components of the cell-wall polysaccharide.[8,9] Although TLR2 and TLR4 with their coreceptor CD14 were originally detected as key components of the pathogen-associated molecular machinery that recognizes gram-positive and gram-negative bacteria, they also identify a wide variety of endogenous structures, including oxLDL.[10,11] TLR signaling initiates cytokine production by activating transcription factor NF-kB (nuclear factor-kappa B) through its adaptor protein MyD88 (myeloid differentiation primary response protein).[12] Thus, the immune responses that have evolved to combat bacterial infections are shared with those involved in the immune response to the endogenous inflammatory components of atherogenesis.[2]

The possibility that infectious agents are involved in the chronic inflammation of coronary plaques has been considered for a long time.[3,13] Reports from the late 1980s on the association of *Chlamydia* antibodies and chronic dental infections with myocardial infarction (MI)[14,15] greatly increased the academic interest in the infection hypothesis that was subsequently tested in large long-term antibiotics trials. Most of these trials failed, as orally administered bactericidal antibiotics effective against *Chlamydia pneumoniae*[16–18] and periodontal bacteria[19] yielded no reduction in the rate of secondary cardiovascular events.[20] Contrasting results in smaller trials were also published.[20–23] Currently, it is known that chronic inflammation is often due to a bacterial biofilm that can evade the innate immune system and is resistant to antibiotics, which possibly may explain the trial failures. A dormant biofilm may be activated by some poorly known factors, executing the production of a new generation of virulent bacteria that break out of the biofilm.[24–26]

Using broad-range 16-S rDNA polymerase chain reaction (PCR), followed by cloning and sequencing, we found in 2005 that coronary atheromas collected at autopsy contained DNA of viridans group streptococci, among other species of bacteria commonly found in the oral cavity.[27] Viridans group streptococcal DNA was also detected in most thrombus aspirates from patients with MI,[28] in ruptured cerebral aneurysms,[29] in thrombus aspirates from surgical patients with lower-limb arterial and deep venous thrombosis,[30] and in cerebral thrombi from patients with acute ischemic stroke treated with thrombectomy[31]; in the last-mentioned series, the presence of viridans streptococci was also confirmed by bacterial immunohistochemistry.[32] The presence of DNA from viridans streptococci has also been confirmed by other studies.[33–35] The recent approach of using 16S rRNA gene sequencing–based metagenomic studies has revealed that atherosclerotic plaques harbor DNA sequences from dozens or even hundreds of

oral, intestinal, skin or environmental bacteria[35,36], suggesting the presence of a microbiome. However, these sequences are mostly present in tiny <1% fractions, the majority of them most probably carried by single or small groups of phagocytes or being contaminants from PCR reagents and other laboratory equipment or environmental elements, with no clinical significance.[37]

Viridans streptococci are harmless commensals in the oral cavity, where they act as initial colonizers in the development of a dental biofilm[38] known as dental plaque. However, they have been found to be the major species of bacteria detected in blood following tooth extraction and other dental procedures[39–41] and are considered to be among the most common pathogenetic agents of infective endocarditis,[42,43] which is a typical example of a chronic bacterial infection due to a bacterial biofilm.[44]

The overriding question is the issue of causality—do the bacteria that can be found in atherosclerotic plaques have any causal role in the development of the plaques, or are they or their DNA present as a consequence of inflammation? Sensitive molecular probes may detect bacterial DNA engulfed by phagocytic cells also originating from a noncardiac site, such as the gingiva, skin, respiratory tract, or intestinal tract.[45] Bacteria might attach as a biofilm onto the uneven surface of the plaques following transient bacteremia with no pathogenic significance. In a real infection, the pathogen invasion activates the innate immune system and there should be evidence of the presence of the agent in an atherosclerotic plaque but not in normal blood vessels.[13] In an autopsy study, this idea may be carried further by studying whether the presence of bacteria in a coronary atheroma is linked with the rupture and thrombosis of the atheroma and with death due to an MI. In this setting, the absence of bacteria should associate with a normal artery or nonruptured atheroma and with a noncoronary cause of death.

In the present study, we aimed to investigate the role of oral bacteria, and particularly that of viridans streptococci, and the innate immune system in the inflammation of atherosclerotic plaques, in addition to examining their role as a risk factor for a fatal MI. This was achieved by first analyzing the presence and prevalence of oral bacterial DNA in atheromas with real-time quantitative PCR (RT-qPCR) and by applying bacterial immunohistochemistry in a unique series of people experiencing sudden out-of-hospital death. We then used bacterial-stimulated TLR cell lines to study in vitro which TLR signaling routes the streptococci and other candidate bacteria preferred to activate. Next, we analyzed immunohistochemically whether receptors of TLR signaling pathways were co-expressed with bacterial immunopositivity in ruptured fibrous caps of coronary atheromas and whether the adaptive immune system was also activated. To confirm the results of our studies on autopsy cases, we also studied the presence of oral bacterial genomes and bacterial immunopositivity in a series of atherosclerotic endarterectomy samples from surgical patients. With these patient samples, we performed a genome-wide expression (GWE) analysis of atherosclerotic plaques to study the up- or downregulation of genes in TLR-dependent signaling pathways for recognizing bacteria.

## METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

### Tampere Sudden Death Study

The TSDS (Tampere Sudden Death Study) comprises a prospective series of 121 autopsies on White men and women aged >35 years, who died out of hospital in Tampere, Finland, and were subjected to a medicolegal autopsy at the Department of Forensic Medicine, Faculty of Medicine and Health Technology, Tampere University during 2010 to 2012 (Table). The Tampere region has a population of approximately 0.3 million. In Finland, a complete medicolegal autopsy is mandated in all cases of unexpected out-of-hospital deaths of an individual with no history of a serious disease or if the individual may have died of unnatural causes. Of the deaths, 57 (47.1%) were due to coronary heart disease (CHD), 39 (32.2%) due to other diseases, and 25 (20.7%) due to nonnatural causes. The TSDS series thus includes a representative selection of sudden prehospital cardiac deaths occurring in this region.

In all cases, the postmortem time was <5 days. Death cases with knowledge on antibiotic treatment were excluded. The autopsy room was ventilated with a negative room pressure airflow exhaust system and the surfaces of the autopsy room were disinfected with overnight UV radiation. The autopsy table is equipped with a downward laminar suction that constantly removes air from the table. At autopsy, coronary samples were obtained aseptically with NaOH-soaked instruments, as described in detail in a subsequent section, and the presence of an old or acute MI with or without coronary thrombosis was studied macroscopically and by microscopy. The cause of death was determined in a routine manner by pathologists who had no knowledge of the analysis of the study results. The TSDS protocol was reviewed and approved by the Ethics Committee of Pirkanmaa Hospital District (Permission number R09097) and the National Supervisory Authority for Welfare and Health.

### Tampere Vascular Study

The vascular sample series from the TVS (Tampere Vascular Study) included femoral arteries, carotid arteries, and abdominal aortas (Table) obtained during

**Table Characteristics of the Study Series.**

| | Tampere Sudden Death Study | Tampere Vascular Study |
|---|---|---|
| No. | 121 | 96 |
| Type of series | Autopsy series | Clinical patients |
| Age, y | 63.1 (16.5) | 69.0 (10.2) |
| Men | 89 (73.6%) | 69 (71.9%) |
| Body mass index, kg/m$^2$ | 29.7 (7.2) | 27.0 (4.4) |
| Cause of death | | |
| Coronary heart disease | 57 (47.1%) | |
| Other disease | 39 (32.2%) | |
| Nonnatural cause | 25 (20.7%) | |
| Origin of artery samples | Autopsy | Surgical operation |
| Coronary samples (left anterior descending+right coronary artery) | 359* | … |
| Carotid endarterectomy | | 29 (30.2%) |
| Femoral endarterectomy | | 24 (25.0%) |
| Abdominal aortic bypass | | 15 (15.6%) |
| Coronary artery bypass (LITA graft) | | 28 (29.2%) |
| AHA classification of atherosclerosis | 359* | 59* |
| Healthy | 0 | 19† (32.2%) |
| Type I | 97 (27.0%) | 1† (1.7%) |
| Type II | 14 (3.9%) | 0 |
| Type III | 57 (15.9%) | 4 (6.8%) |
| Type IV | 11 (3.1%) | 5 (8.5%) |
| Type V | 156 (43.4%) | 14 (23.7%) |
| Type VI | 24 (6.7%) | 16 (27.1%) |
| Bacterial qPCR | 107‡ | 19‡ |
| Immunohistochemistry | 359 | 59 |
| Genome-wide expression analysis | … | 96 |

AHA indicates American Heart Association; LITA, left internal thoracic artery; and qPCR, quantitative polymerase chain reaction.

*In the Tampere Sudden Death Study series the number of coronary samples from left anterior descending and right coronary artery varied between 2 to 4/case, and in the Tampere Vascular Study series 59/96 samples were available for histology and immunohistochemistry,

†Of 20 available LITA grafts,19 were histologically healthy and 1 was AHA Type 1.

‡Available for qPCR; healthy: 2 cases, AHA Type IV: 4 cases, AHA type V: 5 cases and AHA Type VI: 8 cases.

open vascular procedures during 2005 to 2009 from 96 White male and female patients fulfilling the following inclusion criteria: (1) carotid endarterectomy due to symptomatic or asymptomatic and hemodynamically significant (>70%) carotid stenosis, or (2) femoral or (3) aortic endarterectomy with aortoiliac or aortobifemoral bypass due to symptomatic peripheral arterial disease.[46] An exclusion criterion was the patient's refusal to participate in the study. The original purpose of the study was to obtain fresh atherosclerotic and healthy arterial samples for a GWE analysis of the function of genes in different vascular beds.[47] The present series comprises vascular samples obtained under sterile conditions from 96 White men and women, 68 of whom underwent carotid (n=29) or femoral endarterectomy (n=24) or abdominal aortic bypass (n=15). Control samples (n=28) were obtained from the left internal thoracic artery (LITA) during coronary artery bypass due to symptomatic coronary artery disease. All open vascular surgical procedures were performed at the Division of Vascular Surgery and the Heart Center at Tampere University Hospital. The study was reviewed and approved by the Ethics Committee of Pirkanmaa Hospital District (permission number 99204). All clinical investigations were conducted according to the Declaration of Helsinki principles, and the study participants gave informed consent.

The study design was compatible with the Strengthening the Reporting of Observational Studies in Epidemiology guidelines for cross-sectional studies.[48]

## Statistical Analysis

Statistical analyses were performed with version 25 of IBM SPSS statistics software (IBM SPSS Statistics for Windows, version 24.0. IBM Corp. 2016. Armonk, NY). $\chi^2$ or Fisher exact tests were used in statistical comparisons of categorical variables and the Kruskal–Wallis test to calculate differences in continuous variables between the groups. Logistic regression (enter mode) was used to perform age-adjusted analyses.

## Handling and Scoring of the Histological Arterial Samples

In the TSDS, the pericardium was opened aseptically using NaOH+$H_2O$+EtOH–treated instruments and sterile gloves. The heart was placed on a sterile surgical covering, and the coronary arteries were dissected free from the surface of the heart using sterile instruments and stored on sterile Petri dishes, on ice and under cover, until transfer to the laboratory during the same day, where they were washed with 2 mL sterile phosphate buffer. In the laboratory, a transverse section from the most severe or thrombotic coronary plaque from the left anterior coronary artery and the right coronary artery (RCA) were collected for histology under sterile conditions and with sterile instruments. An adjacent section was taken for bacterial RT-qPCR. A histological control sample was taken from the distal healthy or less atherosclerotic sites of the same artery, but the control sample was not taken if the severity of atherosclerosis did not differ from the diseased plaque site. Similarly, if there was no visible atherosclerosis, only 1 sample was taken from a proximal segment

of the RCA and the left anterior coronary artery. Histological samples (n=359) were immediately placed in 6-compartment Tissue-Tech boxes, fixed in 10% buffered sterile formalin for 24 hours and then incubated in sterile EDTA solution for 1 to 2 weeks to decalcify the coronary plaques, and embedded in paraffin.

In the TVS, small 2×2 to 3 mm endarterectomy or LITA samples were immediately cut into 2 parts with a sterile scalpel: a histological sample was placed in sterile 10% buffered formalin, and the other section of the sample that was to be used for GWE analysis was immediately soaked in a sterile tube containing RNALater solution and transferred to the laboratory for further handling. From the initial 96 samples obtained for histology, 59 (61.5%) were large enough and contained arterial tissue layers besides calcification for assessing the grade of atherosclerosis and for immunohistochemical studies.

Paraffin blocks were stored in closed cardboard boxes in a storage room at a constant temperature of 17 °C. Decalcified coronary and endarterectomy samples were processed onto slides as usual, stained with the Verhoeff HE (hematoxylin - eosin) method, and classified according to the terminology of the American Heart Association (AHA).[49] In the TSDS, there were no completely healthy samples because even macroscopically normal-looking samples microscopically showed at least intimal thickening (early atherosclerotic lesion, Type I).

## Bacterial RT-qPCR

In the TSDS series, samples obtained from the proximal RCA for RT-qPCR were transferred into an Eppendorf tube and stored at –70 °C. DNA was extracted using standard methods and was successful in 107 (88.3%) of the 121 proximal RCA samples. DNA extracted from 200 µL of the 2 mL of sterile phosphate buffer used to rinse the artery was used as a control reference sample. As described in detail elsewhere,[28] oligonucleotide primers and probes for RT-qPCR, designed using Primer Express 1.5 software (Applied Biosystems, USA), were used to confirm/exclude the presence of possible candidate bacteria species from the oral cavity, respiratory tract, gut, and skin, such as typical periapical abscess bacteria (*Streptococcus spp.*/mainly *Streptococcus mitis* group, *Streptococcus anginosus* group, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Prevotella intermedia*, and *Parvimonas micra*), periodontal bacteria (*Porphyromonas gingivalis, Aggregatibacter [néé Actinobacillus] actinomycetemcomitans*, *Fusobacterium nucleatum*, *Dialister pneumosintes*, and *Treponema denticola*), as well as *Chlamydia pneumoniae*. The specificity and cross-reactivity of primers and probes were tested as reported earlier,[28] that is, against human DNA, bacteria cultures from clinical samples, as well as against reference bacteria from the American Type Culture Collection (ATCC) biological materials resource collection (https://www.lgcstandards-atcc.org/en/Products/Cells_and_Microorganisms/Bacteria.aspx); *Streptococcus mitis* ATCC 49456, *Streptococcus sanguinis* ATCC 10556, *Streptococcus gordonii* ATCC 10558, *Streptococcus anginosus* ATCC 33397, *Prevotella intermedia* ATCC 25611, *Treponema denticola* ATCC 35405, *Dialister pneumosintes* ATCC 33048, *Fusobacterium nucleatum* ATCC 25586, *Parvimonas micra* ATCC 33270, *Aggregatibacter actinomycetemcomitans* ATCC 700685, *Porphyromonas gingivalis* ATCC 33277, *Chlamydophilia [néé Chlamydia] pneumoniae* ATCC 53592 (LCG Standards AB, Borås, Sweden). The cutoff values (ie, detection limits) were 40 cycles for all candidate bacteria, except for *Streptococcus* spp., mainly *Streptococcus mitis* group and *Fusobacterium nucleatum*, whose cutoff cycle was 35. Universal bacterial primers and probes were used to evaluate sample positivity. The cutoff for universal bacterial measurement was 31 cycles. The amount of human DNA was detected by measuring a housekeeping gene, RNaseP (Applied Biosystems, USA), and used as a reference. All primers and probes have been described elsewhere.[28] Amplification and detection were performed using the ABI PRISM 7900 sequence detection system (Applied Biosystems, USA). The relative amounts of these organisms in specimens were calculated by the comparative *Ct* method (ΔΔCt, $\Delta Ct_{sample} - \Delta Ct_{reference\ sample}$) as previously described.[28]

From the TVS series, 17 calcified atherosclerotic plaques and 2 healthy control LITA samples obtained aseptically during surgical procedures were available for bacterial RT-qPCR due the fact that most of the small-sized samples were already used for GWE analysis and histology. The cutoff values for these clinical samples were 40 cycles for all candidate bacteria, except for *Streptococcus* spp., mainly the *Streptococcus mitis*-group and *Fusobacterium nucleatum*, whose cutoff cycle was 37. For universal bacterial measurements, the cutoff value was 33 cycles.

## In Vitro Culture of TLR Cell Lines

Human embryonic kidney 293 cell line cells stably transfected with either the empty plasmid (293-Null) or human TLR1/2, TLR2/6, or TLR4/MD2/CD14 genes were purchased from InvivoGen and maintained in DMEM (InvitroGen), supplemented with 4.5 g/L glucose, 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 10 µg/mL blasticidin S (InvivoGen) for 293-Null, 293-hTLR1/2, and 293-hTLR2/6; and with 25 µg/mL Hygromycin B (InvivoGen) for 293-hTLR4/MD2/CD14(NA, TP, CM). Viability was monitored with the use of 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium

(MTT; Sigma). For stimulation experiments, 3×105 stable transfected cells were seeded into a 48-well plate in a total volume of 300 μL of complete medium and allowed to adhere overnight. The following day, fresh medium was added, and the cells were stimulated with either inactivated *Streptococcus mitis*, *Streptococcus sanguinis*, *Streptococcus gordonii*, *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, or *Chlamydophilia pneumoniae* from the ATCC stock culture collection (LCG Standards AB, Borås, Sweden; 107 or 106 or 105/mL), or with TNFa (tumor necrosis factor alpha;10 ng/mL; InvivoGen, Source BioScience LifeSciences, UK), the Pam3CSK4 lipopeptide (10 ng/mL; InvivoGen), or *Escherichia coli* LPS (100 ng/mL; Sigma-Aldrich) or FSL-1 (100 μg/mL; InvivoGen) for 6 hours. The protocols of stimulation with TLR ligands have been described in detail previously.[11] NF-kB activation was assessed via the NF-kB luciferase reporter assay (Bright-Glo Luciferase Assay System, Promega, UK). Each condition was tested in triplicate cultures, and each triplicate was analyzed separately.

## Immunohistochemical Methods

In-house antibodies to *Streptococcus mitis*, *Streptococcus gordonii*, and *Streptococcus sanguinis* were obtained by immunizing mice with a standard immunization protocol and confirmed by a chemiluminescent immunoassay[50] using the original immunogens as test antigens. Methods fulfilled the *Animal Research: Reporting of In Vivo Experiments* guidelines.[51] In preliminary immunohistochemical stainings, the antibodies showed cross-reactivity due to structural similarities of the capsular structure,[52] especially among different viridans streptococci (Figures S1 and S2). These antibodies were pooled together (1:1:2) for immunohistochemistry. The expression of bacteria-recognizing receptors and adaptive immune responses were studied using CD68 (Nordic Biosite), CD14 (Novocastra), TLR2 (Abcam), TLR4 (Abcam), and NF-kB (Abgent). Cases of a ruptured coronary atheroma with thrombosis and control samples from the distal "healthy" site of the same artery were also immunostained with MyD88 (Nordic Biosite), CD3 (Nordic BioSite), and CD3z (CD247) (ABD-Serotec) antibodies, as well as with a preimmune serum cocktail from mice that were immunized with viridans streptococci strains. The specificity of immunostainings was studied by staining the same samples with antibodies to beta hemolytic Group A (*Streptococcus pyogenes*; Abcam), Group B (*Streptococcus agalactiae*; Abcam), *Escherichia coli* (Santa Cruz Biotechnology), and IgG1 isotype (Invitrogen), using the same concentrations. Antibodies were diluted in Dako REAL antibody diluent (S2022), pipetted onto slides for 50 minutes, and washed in TBS-Tween for 2 to 5 minutes. Secondary staining was performed with the Dako REAL EnVision Detection System (K5007) and visualized with DAB according to the kit protocol. To exclude the possibility of an erroneous staining result due to endogenous peroxidase activity or necrotic cells/tissue, confirmatory staining was performed where the primary antibody was replaced with a dilute, as well as with DAB only. Samples were also stained with preimmunization serum from the same mice.

For visualizing immunopositive areas in photographs, we applied ImageJ software to create color-convoluted images[53,54] of the arteries (Figures S3 and S4).

The streptococcal immunopositivity of the coronary plaque samples was scored as 0=no positivity; slight (+)=scattered viridans streptococcal immunopositive inflammatory phagocytes or small streptococcal immunopositive areas; moderate (++)=moderate numbers of streptococcal immunopositive phagocytes or larger biofilm areas; and severe (+++)=numerous immunopositive inflammatory cells or larger biofilm areas, or free bacterial infiltrates. In a subset analysis of color convoluted images using ImageJ software, the mean surface area percentages of immunopositive areas were: 0=0%; +=1.8%; ++=2.7% and +++=8.7% of the sample area at the magnification of 10× and with the same threshold value.

## RNA Isolation and GWE Analysis in the TVS

Fresh endarterectomy samples were immediately soaked in RNALater solution, homogenized using an Ultra-TurraxR T80 homogenizer, and stored at +8 °C. The RNA was extracted within 14 days with the Trizol reagent and the miRNEasyR Mini-Kit with the RNase-Free DNase Set, according to the manufacturers' instructions. The concentration and quality of RNA were evaluated spectrophotometrically (BioPhotometer, Eppendorf, Wesseling-Berzdorf, Germany). The gene set enrichment analysis microarray experiments were performed in 2009 with an Illumina HumanHT-12 v3 Expression BeadChip (Illumina). In brief, 300 to 500 ng of RNA was reverse-transcribed into cRNA and biotin-UTP-labeled using the Illumina TotalPrep RNA Amplification Kit (Ambion); 1.500 ng of cRNA was then hybridized onto the Illumina HumanHT-12 v3 Expression BeadChip. The BeadChips were scanned with the Illumina iScan system. After background subtraction, raw intensity data were exported using the Illumina GenomeStudio software. Further data processing was conducted by means of R language and appropriate Bioconductor modules. The accuracy of Illumina HumanHT-12 v3 Expression BeadChip microarray methodology in measuring the gene expression was verified previously by real-time quantitative TaqMan PCR with samples from the TVS.[55]

We applied a locally estimated scatterplot smoothing normalization method implemented in the R/Bioconductor package Lumi (www.bioconductor.org). Locally estimated scatterplot smoothing normalization was selected for the data because it yielded the best accuracy in comparison with the quantitative reverse transcription polymerase chain reaction data for artery samples. Quality control was performed using sample clustering and multidimensional scaling. Seven outliers were removed due to low expression profiles, 4 from the carotid artery group and 3 from the LITA group. The Bioconductor module was also used for single-probe analysis, including fold-change calculations and filtering the probes. The statistical analysis was carried out using the Limma package (www.bioconductor.org). Pathway analysis of the expression data (all diseased versus controls) was performed using the gene set enrichment analysis implemented with the GSEA Java desktop application and MsigDB (Molecular Signature Database), version 3.0. Statistical analysis was performed using SPSS version 19 (IBM Co, USA). The nonparametric Mann–Whitney *U* test with post hoc correction was used for comparing mRNA expression between atherosclerotic and control tissues and for finding differently expressed genes. The results are presented as an average fold change. The averaging was done for each arterial bed. The selection criteria were a 2.0-fold change in gene expression and a *P* value of <0.05. With these criteria, the specificity of the microarray data was found to be 95% in comparison to qRT-PCR. The description of the methods is compatible with the guidelines of Minimum Information about a Microarray Experiment.[56]

# RESULTS

## Presence of Bacterial DNA in Atherosclerotic Plaques

Bacterial DNA was detected by RT-qPCR in 65.7% of the coronary plaque samples from the TSDS autopsy series and in 57.9% of the available surgical samples from the TVS series, using universal bacterial primers and probes.

Bacterial DNA profiles were similar in both series (Figure 1A). The most common bacterial DNA detected in both series belonged to oral viridans group streptococci (*Streptococcus* spp. mainly *Streptococcus mitis* group), which were found in 42.1% of the autopsy-obtained coronary plaques and in 42.9% of the surgical samples. Streptococcal DNA positivity was detected in 35.0% of the proximal RCA samples with AHA type I or II (early atherosclerosis), in 52.6% in samples with AHA type III or IV (mild to moderate atherosclerosis), in 42.1% in samples with AHA type V (fibroatheromas or partially calcified plaques), and in 60% in samples with AHA type VI (plaque rupture and thrombosis/hemorrhage), showing a nonsignificant trend (*P*=0.246). DNA from other oral bacteria (*Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, and *Prevotella intermedia*) was also found in similar frequencies in both series. *Chlamydia pneumoniae* DNA was detected in 3.8% of the TSDS samples and in 10.5% of the clinical TVS samples.

## Viridans Streptococcal Immunopositivity and Severity of Atherosclerosis

We raised in-house antibodies to the 3 most common viridans streptococci (*Streptococcus mitis, sanguinis*, and *gordonii*) and pooled these together for immunohistochemistry (Data S1, Figures S1 and S2). Using this antibody pool, immunopositivity was detected in 217 (60.4%) of the 359 coronary artery samples obtained from the 121 cases of the TSDS autopsy series, and in 31 (52.5%) of the 59 samples available from the clinical TVS series of patients with symptomatic cerebrovascular or peripheral arterial disease.

In both series, viridans streptococcal immunopositivity score showed a similar, strong association with the severity of atherosclerosis, scored using the American Heart Association classification[49] (Figure 1B).

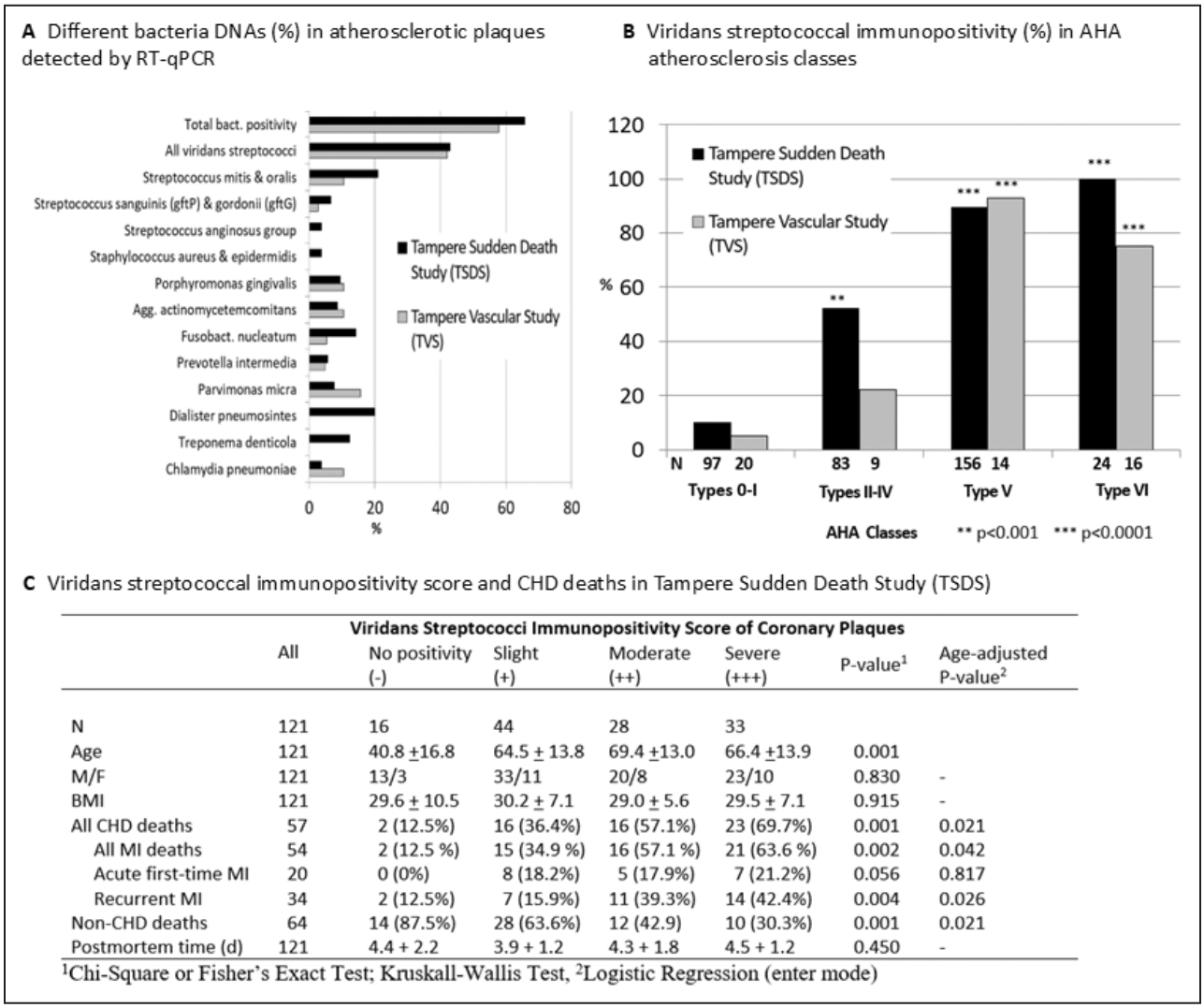
## Viridans Streptococcal Immunopositivity and Death Due to CHD and MI

In univariate analysis, the immunopositivity score associated highly significantly with age (*P*=0.0001), all deaths due to CHD with or without an MI (*P*=0.0001), all MI deaths (*P*=0.002), and deaths due to a recurrent MI (*P*=0.004). Immunopositivity tended to associate with acute first-time MI (*P*=0.056) but not with sex, body mass index, or postmortem time (Figure 1C). Associations of the immunopositivity score with deaths due to CHD, all MIs, and recurrent MIs remained significant after adjustment for age.

In the TSDS series, the immunopositivity score correlated with the presence (*P*=0.007) and relative amount (*P*=0.005) of viridans streptococcal DNA in RT-qPCR. In the clinical TVS series, the correlation with RT-qPCR results could not be calculated because there were only 8 cases with both immunohistochemistry and qPCR performed on the samples due to the low number of endarterectomy samples available for RT-qPCR.

## Viridans Streptococcal Biofilm in Advanced Autopsy Coronary Plaques and Clinical Endarterectomy Specimens

In the TSDS series, there were no completely normal coronary arteries due to the age limit (>35 years) of the




C Viridans streptococcal immunopositivity score and CHD deaths in Tampere Sudden Death Study (TSDS)

| | | Viridans Streptococci Immunopositivity Score of Coronary Plaques | | | | | |
|---|---|---|---|---|---|---|---|
| | All | No positivity (-) | Slight (+) | Moderate (++) | Severe (+++) | P-value[1] | Age-adjusted P-value[2] |
| N | 121 | 16 | 44 | 28 | 33 | | |
| Age | 121 | 40.8 ±16.8 | 64.5 ± 13.8 | 69.4 ±13.0 | 66.4 ±13.9 | 0.001 | |
| M/F | 121 | 13/3 | 33/11 | 20/8 | 23/10 | 0.830 | - |
| BMI | 121 | 29.6 ± 10.5 | 30.2 ± 7.1 | 29.0 ± 5.6 | 29.5 ± 7.1 | 0.915 | - |
| All CHD deaths | 57 | 2 (12.5%) | 16 (36.4%) | 16 (57.1%) | 23 (69.7%) | 0.001 | 0.021 |
| All MI deaths | 54 | 2 (12.5 %) | 15 (34.9 %) | 16 (57.1 %) | 21 (63.6 %) | 0.002 | 0.042 |
| Acute first-time MI | 20 | 0 (0%) | 8 (18.2%) | 5 (17.9%) | 7 (21.2%) | 0.056 | 0.817 |
| Recurrent MI | 34 | 2 (12.5%) | 7 (15.9%) | 11 (39.3%) | 14 (42.4%) | 0.004 | 0.026 |
| Non-CHD deaths | 64 | 14 (87.5%) | 28 (63.6%) | 12 (42.9) | 10 (30.3%) | 0.001 | 0.021 |
| Postmortem time (d) | 121 | 4.4 + 2.2 | 3.9 + 1.2 | 4.3 + 1.8 | 4.5 + 1.2 | 0.450 | - |

[1]Chi-Square or Fisher's Exact Test; Kruskall-Wallis Test, [2]Logistic Regression (enter mode)

**Figure 1. Occurrence of bacterial DNAs and association of streptococcal immunopositivity with atherosclerosis and death due to CHD.**

**A**, A comparison of the frequencies (%) of different bacterial DNA shows similar profiles in the atherosclerotic plaques of the TSDS autopsy coronary samples (black bar) and the TVS clinical endarterectomy samples (gray bar). **B**, Positive viridans group streptococcal immunostaining (%) among the TSDS autopsy coronary samples (black bar) and TVS clinical endarterectomy specimens (gray bar) shows a highly significant association in both series with the severity of atherosclerosis, using the score defined by the American Heart Association. **C**, The viridans streptococcal immunopositivity score in the TSDS autopsy series associated with death due to CHD and MI along with age but did not associate with postmortem time, sex, or BMI. AHA indicates American Heart Association; BMI, body mass index; CHD, coronary heart disease; MI, myocardial infarction; RT-qPCR, real-time quantitative polymerase chain reaction; TSDS, Tampere Sudden Death Study; and TVS, Tampere Vascular Study.

series. Of the 97 artery segments with intimal thickening (AHA type I), 11 (11.3%) were immunopositive, occurring mainly in phagocytes in the adventitial *vasa vasorum*, but most of these coronaries were immunonegative (Figure 2A through 2C). Viridans streptococcal immunopositivity was detected in 43 (51.8%) of the 83 plaques with more advanced atherosclerotic lesions comprising foam cells, extracellular subintimal lipid pools, or a lipid core (AHA types II–IV), mainly presenting as subintimal phagocytic infiltrates. Of the 156 fibroatheromas or partially calcified plaques with no rupture or hemorrhage (AHA type Va–c), 140 (89.7%) were immunopositive, showing mainly biofilm-like colonization (Figure 2D through 2F and 2S through 2V). Biofilms were not recognized by macrophage marker CD68, suggesting that they evade the immune system (Figure 2W through 2Z).

In all 24 cases of MI due to complicated coronary plaques with a rupture or thrombosis/hemorrhage (AHA type VI), the plaques were infiltrated by immunopositive viridans streptococci, localizing inside macrophages or freely at the rupture site (Figure 2G through 2I; Figure 3H

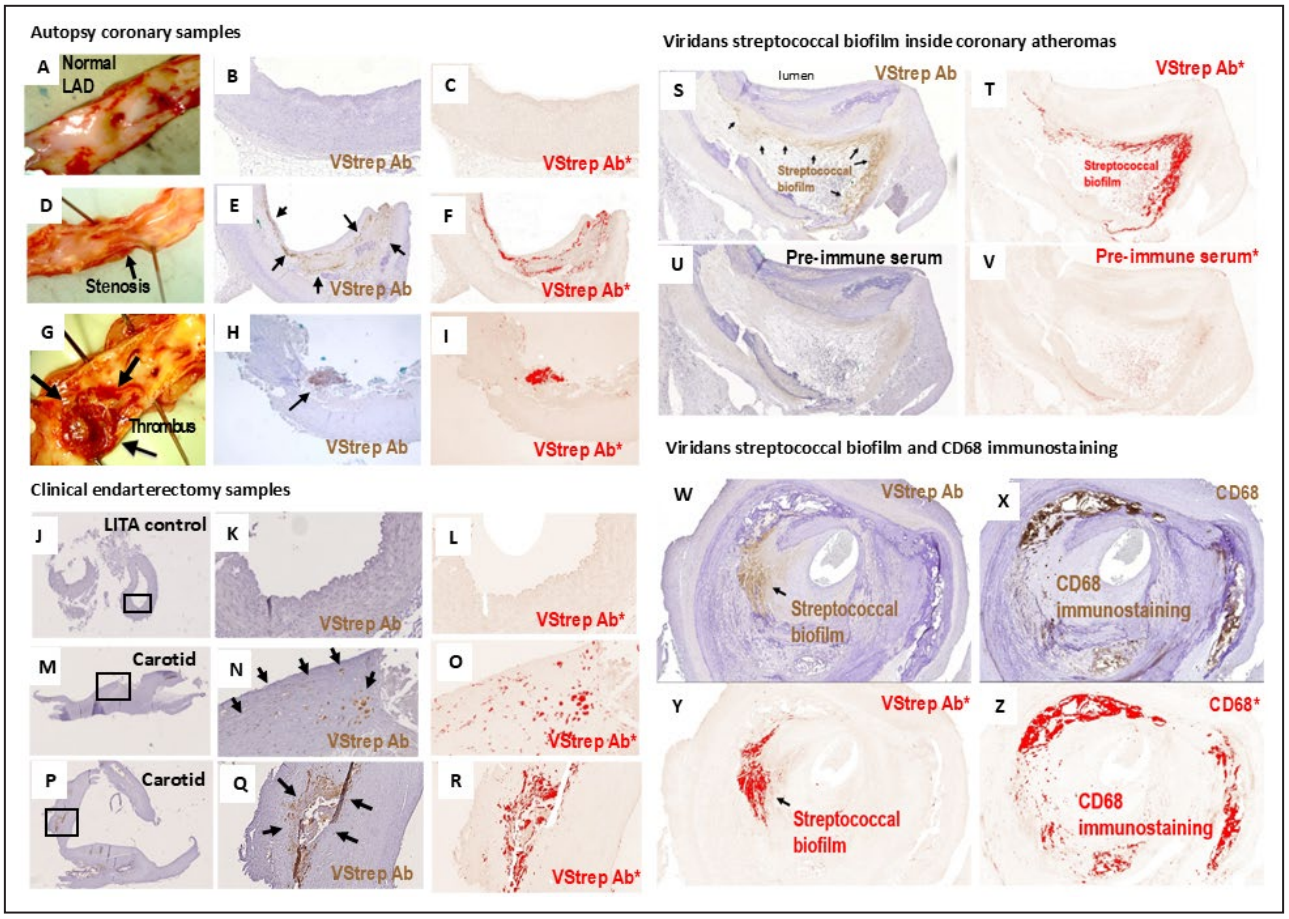



**Figure 2. Immunostaining (1:2000) with pooled viridans streptococci antibodies (brown) and ImageJ software–created color convoluted images (red*) of coronary arteries in the TSDS autopsy series and in the carotid endarterectomy and left internal thoracic control samples in the TVS clinical series.**

**A** through **C**, Coronary artery with intimal thickening without streptococcal immunopositivity. **D** through **F**, Narrowed fibrocalcific plaque with biofilm-type immunoreactivity inside the plaque and in the subintima over the plaque (arrows). **G** through **I**, Rupture and thrombosis (arrows) of a soft thin-walled coronary atheroma (left) with fatal coronary thrombosis. The remnants of the ruptured fibrous cap are stained by a viridans streptococcal antibody cocktail (arrow). **J** through **L**, A control LITA artery sample taken during coronary bypass shows no immunopositivity. **M** through **O**, Carotid endarterectomy samples have immunopositive infiltrates (arrows) in the fibrous capsule. **P** through **R**, Streptococcal biofilm (arrows) inside the necrotic core of a carotid atheroma. **S** through **T**, A viridans streptococcal antibody–positive biofilm borders the lipid core of a coronary atheroma (arrows). **U** through **V**, Preimmune serum (1:500) originating from the same mouse before raising antibodies does not stain the biofilm. **W** through **Z**, Staining of a tight coronary stenosis site with a viridans streptococcal antibody and macrophage marker CD68 shows that macrophages do not detect bacterial biofilms (arrow). CD68 stains cholesterol-containing areas (arrows) harboring macrophage-derived foam cells. Ab indicates antibody; LAD, left anterior descending coronary artery; LITA, left internal thoracic artery; TSDS, Tampere Sudden Death Study; TVS, Tampere Vascular Study; and VStrep, viridans streptococcus.

through 3L) but often seen as a diffuse biofilm-like colonization at the rupture site of the fibrous cap of atheroma (Figure 4E through 4G).In the surgical specimens of the TVS series (Figures 2J through 2O, 3E through 3G), viridans streptococcal immunopositivity was seen in macrophages infiltrating the fibrous cap tissue of the endarterectomy samples and as biofilms inside atheroma remnants. Among the 59 samples, 13 (92.9%) of 14 fibrocalcific atheromas (type V) and 12 (75%) of 16 atheromas with a plaque rupture and thrombosis/hemorrhage (AHA type VI) were immunopositive. Of the 20 LITA grafts, 1 (5%) was immunopositive, showing intimal thickening (AHA type I) and a few immunopositive phagocytes.

## Dispersion of Streptococci Into the Fibrous Caps of an Atheroma

CD68-positive macrophages did not detect the biofilm inside a coronary atheroma (Figure 3A through 3D) but colocalized with groups of discrete cells that were strongly immunopositive for the viridans streptococcal antibody pool. These cells most probably represent virulent streptococci that have been dispersed

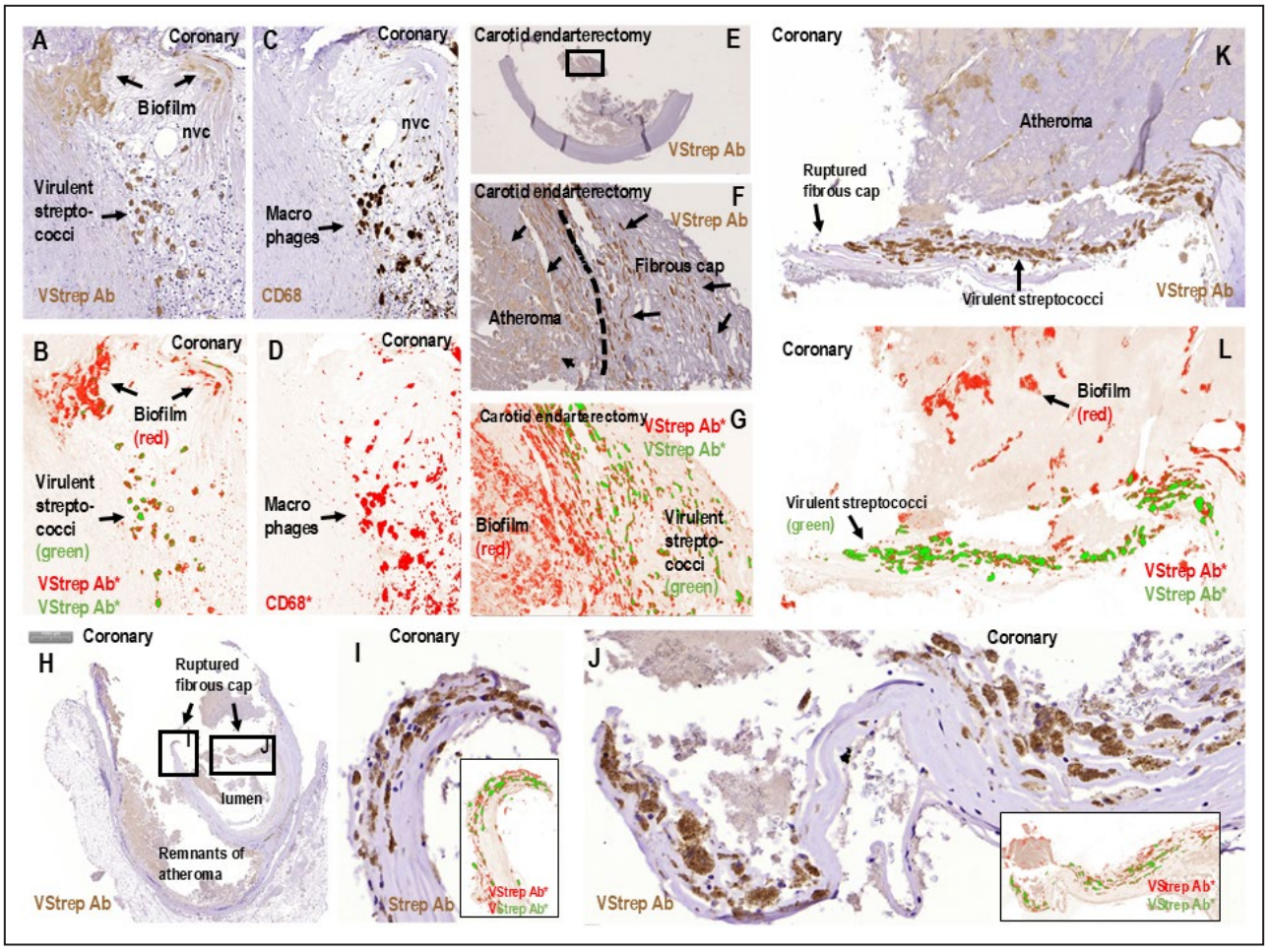



**Figure 3. Immunohistochemical studies on streptococcal biofilms and dispersed virulent streptococci, using conventional peroxidase staining (brown) and ImageJ software to separate the DAB channel with color convolution and to threshold it into red* (biofilm) or green* (virulent streptococci), depending on the intensity of staining (Data S1).**

**A** and **B**, A coronary atheroma of a patient who experienced SCD showing a streptococcal biofilm (red*), along with several dispersed virulent streptococci that express intense staining of the capsule (green*). A neovascular channel (nvc) is seen near the biofilm. **C** and **D**, CD68-positive macrophages do not detect bacterial biofilms but do recognize discrete virulent streptococci. **E** through **G**, An endarterectomy sample from a symptomatic carotid atherosclerotic plaque, showing a streptococcal biofilm (red*) inside the atheroma (dashed line between atheroma and fibrous cap), along with discrete strongly stained streptococci (green*) that have been dispersed from the biofilm and have infiltrated the fibrous cap of the atheroma. **H** through **J**, Ruptured coronary atheroma of another patient who experienced SCD with a myocardial infarction, showing infiltration of the rupture site by strongly stained virulent streptococci (green*) that seem to be largely engulfed by macrophages but also occur as free infiltrates. **K** and **L**, Ruptured coronary atheroma showing a bacterial biofilm inside the atheroma (red*) and strongly stained virulent streptococci (green*) infiltrating the fibrous cap. Ab indicates antibody; nvc, neovascular channel; SCD, sudden cardiac death; and VStrep, viridans streptococcus.

from the biofilm[24,25,57] and detected and engulfed by macrophages. In carotid endarterectomy samples (Figure 3E through 3G), as well in ruptured coronary atheromas (Figure 3H through 3L), biofilms coexisted with these strongly immunopositive cells. As our in-house antibody cocktail was raised against planktonic capsular streptococci strains, it is plausible that this intense staining is due to streptococci expressing capsular virulence factors, in contrast to "sessile" transparent bacteria inside the biofilm, which have a reduced capsule due to downregulated capsular polysaccharide production.[58] The production of a capsule makes them capable of invading the fibrous cap of an atheroma after being released from the biofilm. The weaker staining of the biofilm suggests that there may be capsular proteins or cellular debris released from dead bacteria[58] mixed with the extracellular matrix.

## Stimulation of Toll-Like Receptor Cell Lines With Bacteria

To study the preferred signaling routes of gram-positive and gram-negative bacteria detected by qPCR, TLR cell lines[11] were induced (Figure 4A) by *Streptococcus mitis*, *Streptococcus sanguinis*, and *Streptococcus gorgonii*, as well as *Porphyromonas gingivalis*, *Aggregatibacter*

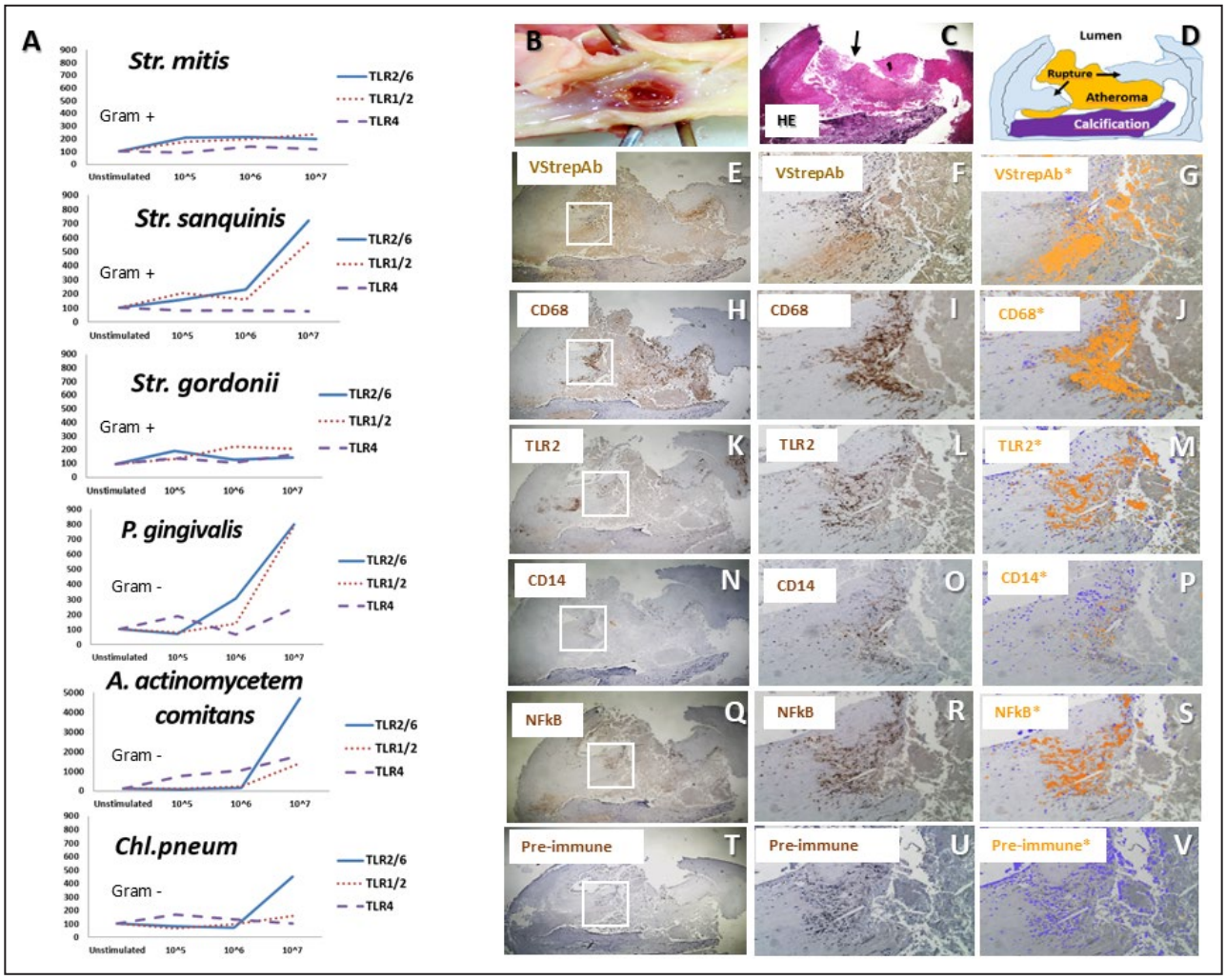



**Figure 4. TLR preferences of different oral bacteria and *Chlamydia pneumoniae* with demonstration of active TLR2 dependent bacterial recognition pathway in ruptured coronary atheroma.**
**A**, Stimulation of HEK-TLR cell lines with reference bacteria. Reference bacteria were heat-inactivated before being added into cultures and cultured as triplicates. Mean values of NF-kB luciferase activities are presented as % medium values. **B**, Fatal rupture of an atheroma in the left anterior descending coronary artery in a 67-year-old man. The occluding thrombus is removed over the rupture site. **C**, HE-stained histological cross-section from the ruptured atheroma, with the contents of the atheroma (arrow) pouring out. **D**, A diagram of the tissue components of the ruptured atheroma. **E** through **G**, Positive immunostaining of the rupture site with a pooled streptococcal antibody cocktail (1:2000), showing biofilm-like staining. **H** through **J**, Macrophage marker CD68; **K** through **M**, Toll-like receptor 2; **N** through **P**, CD14; **Q** through **S**, NFkB. **T** through **V**, Negative staining with preimmune serum (1:1000). The middle column represents a detail of the ruptured fibrous cap from the left column (marked with a white square). The right column represents pseudo-colored images created with ImageJ software to visualize DAB–stained (yellow*) and HE-stained nuclei regions (blue) in the original photos. Ab indicates antibody; HE, hematoxylin and eosin; HEK, human embryonic kidney; NFkB, nuclear factor kappa B; TLR, toll-like receptor; and VStrep, viridans streptococcus.

*actinomycetemcomitans*, and *Chlamydophilia pneumonia*. The most commonly activated signaling route among gram-positive streptococci strains was TLR2 (together with TLR1 or TLR6) at all bacteria concentrations. *Streptococcus sanguinis* showed the strongest activity with an increasing bacterial concentration. In contrast, all gram-negative bacteria (*Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, and *Chlamydophilia pneumoniae*) mainly activated TLR4 in lower bacterial concentrations but began to prefer TLR2 signaling when the bacterial concentration increased.

## Viridans Streptococcal Immunopositivity and Activity of the Innate and Adaptive Immune System

Next, we studied whether the bacteria-recognizing receptors of the TLR signaling pathway were co-expressed with streptococci immunopositivity.

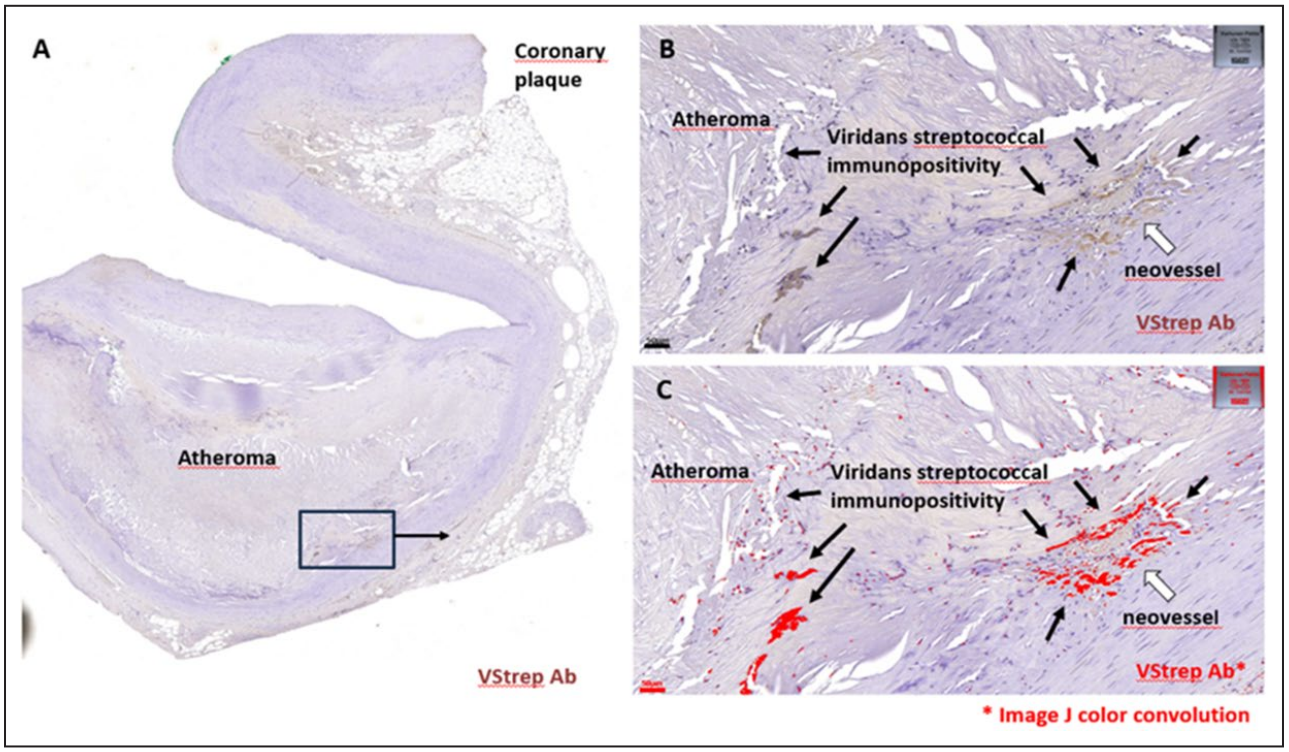



**Figure 5. Entry of viridans group streptococci into coronary plaque may occur through neovessels.**
**A** through **C**, A neovascular thin-walled channel filled with erythrocytes and surrounded by a layer of viridans streptococcal immunopositivity is seen at the boundary between adventitia and media near a large fibrocalcific atheroma of a 61-year-old man, who died of recurrent myocardial infarction. There were also immunopositive infiltrates at the border of the atheroma. The coronary sections were stained with viridans streptococcal antibody cocktail. More contrast was achieved by using the color (red) convolution option of the ImageJ software. Ab indicates antibody; and VStrep, viridans streptococcus.

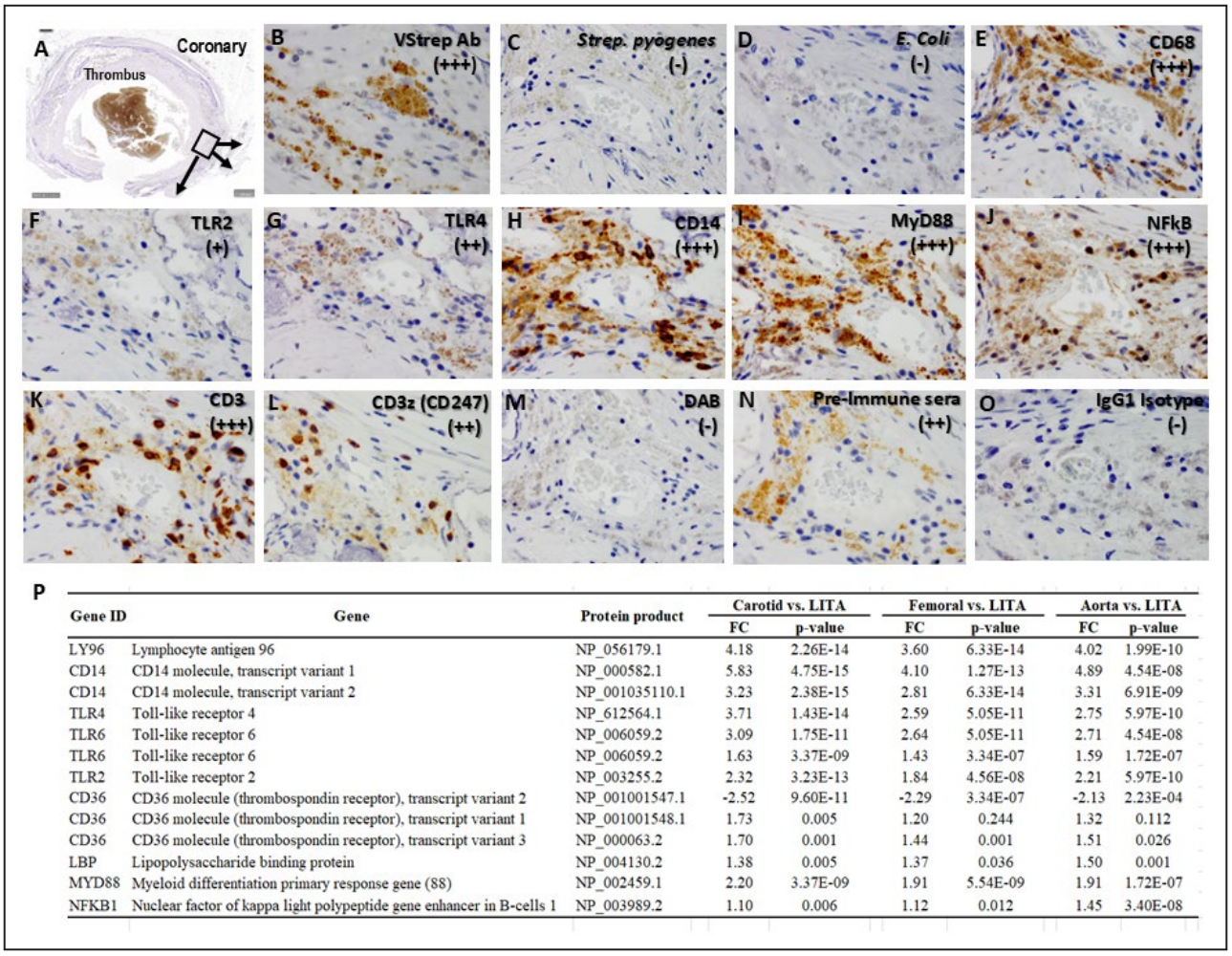
Immunohistochemical staining of ruptured coronary atheromas (Figure 4B through 4V) showed a strong expression of streptococcal immunopositivity in connection with positive staining by antibodies against macrophage marker CD68, cell surface transmembrane TLR pathway signaling proteins TLR2, CD14, and TLR4, as well as ubiquitous transcription factor NF-kB—all at the same rupture site. TLR2 expression was seen clearly, but CD14 and TLR4 (not shown in the Figure 4) were only barely positive, although CD14 is also a coreceptor for TLR2.[12,59] Control staining with preimmune serum was negative. This suggests that streptococcal infiltration of the rupture site had activated a downstream signaling pathway required for the induction of inflammatory cytokines.

The entry of viridans group streptococci into coronary plaque may occur through neovessels (Figure 5A through 5C), that are formed due to the development of hypoxia inside the growing atheroma.[60]

We then studied whether the adaptive immune system was also activated along with the innate immune response in a ruptured coronary atheroma (Figure 6A through 6O). We stained another thrombosed coronary with the viridans streptococcal antibody cocktail, with CD68, TLR2, and TLR4, as well as with MyD88 (intracellular adapter protein activating transcription factor for NF-kB) and NF-kB. These all stained the same bacteria-like subcellular particles inside the macrophages, suggesting an active innate immune response. There was a weak staining with TLR2 and a more intensive staining with TLR4. T-cell markers CD3 and CD3z (CD 247), which together form the T-cell receptor complex on the surface of T cells, were positive, suggesting an ongoing adaptive immune process at the same location. There was also intense staining of the particles with nonimmune serum that was compatible with IgG-mediated opsonization of streptococci.[61] Staining with monoclonal antibodies to beta-hemolytic group A streptococci (*Streptococcus pyogenes*) and *Escherichia coli* was negative. DAB staining was also negative, excluding the presence of necrotic cells. Immunostaining using IgG1 isotype control was negative, suggesting the specificity of immunostainings.

## GWE Studies and Pathway Analysis in the TVS Series

A GWE array on the surgical atherosclerotic plaques from the TVS series comprising clinical vascular surgery patients showed that genes in the metabolic pathways




P

| Gene ID | Gene | Protein product | Carotid vs. LITA | | Femoral vs. LITA | | Aorta vs. LITA | |
|---|---|---|---|---|---|---|---|---|
| | | | FC | p-value | FC | p-value | FC | p-value |
| LY96 | Lymphocyte antigen 96 | NP_056179.1 | 4.18 | 2.26E-14 | 3.60 | 6.33E-14 | 4.02 | 1.99E-10 |
| CD14 | CD14 molecule, transcript variant 1 | NP_000582.1 | 5.83 | 4.75E-15 | 4.10 | 1.27E-13 | 4.89 | 4.54E-08 |
| CD14 | CD14 molecule, transcript variant 2 | NP_001035110.1 | 3.23 | 2.38E-15 | 2.81 | 6.33E-14 | 3.31 | 6.91E-09 |
| TLR4 | Toll-like receptor 4 | NP_612564.1 | 3.71 | 1.43E-14 | 2.59 | 5.05E-11 | 2.75 | 5.97E-10 |
| TLR6 | Toll-like receptor 6 | NP_006059.2 | 3.09 | 1.75E-11 | 2.64 | 5.05E-11 | 2.71 | 4.54E-08 |
| TLR6 | Toll-like receptor 6 | NP_006059.2 | 1.63 | 3.37E-09 | 1.43 | 3.34E-07 | 1.59 | 1.72E-07 |
| TLR2 | Toll-like receptor 2 | NP_003255.2 | 2.32 | 3.23E-13 | 1.84 | 4.56E-08 | 2.21 | 5.97E-10 |
| CD36 | CD36 molecule (thrombospondin receptor), transcript variant 2 | NP_001001547.1 | -2.52 | 9.60E-11 | -2.29 | 3.34E-07 | -2.13 | 2.23E-04 |
| CD36 | CD36 molecule (thrombospondin receptor), transcript variant 1 | NP_001001548.1 | 1.73 | 0.005 | 1.20 | 0.244 | 1.32 | 0.112 |
| CD36 | CD36 molecule (thrombospondin receptor), transcript variant 3 | NP_000063.2 | 1.70 | 0.001 | 1.44 | 0.001 | 1.51 | 0.026 |
| LBP | Lipopolysaccharide binding protein | NP_004130.2 | 1.38 | 0.005 | 1.37 | 0.036 | 1.50 | 0.001 |
| MYD88 | Myeloid differentiation primary response gene (88) | NP_002459.1 | 2.20 | 3.37E-09 | 1.91 | 5.54E-09 | 1.91 | 1.72E-07 |
| NFKB1 | Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 | NP_003989.2 | 1.10 | 0.006 | 1.12 | 0.012 | 1.45 | 3.40E-08 |

**Figure 6. The wall of a ruptured coronary atheroma with occluding thrombosis (A) in the Tampere Sudden Death Study series showed strong staining (B) with the Viridans streptococcal antibody cocktail (1:1000).**
Staining with *Streptococcus pyogenes* mAb (**C**) and *Escherichia coli* mAb (**D**) was negative. Viridans streptococcal immunopositivity was colocalized with CD68-positive macrophages (**E**) and bacteria-recognizing receptors of the TLR signaling pathway (**F** through **J**), as well as with lymphocytes expressing T cell markers CD3 and CD3z (CD 247), which together form a T cell receptor complex on the surface of T cells, indicating the activation of the adaptive immune system (**K** and **L**). DAB staining to detect necrotic cells was negative (**M**), whereas preimmune sera (1:1000) nonspecifically detected streptococci (**N**). Control staining using IgG1 isotype (1:500) was negative (**O**). Genome-wide expression analysis (**P**) on the activity of the signaling pathways of germline-encoded pattern recognition receptors for gram-positive and gram-negative bacteria in atherosclerotic plaque samples from the Tampere Vascular Study in comparison to healthy controls. Ab indicates antibody; FC, fold change; LITA, left internal thoracic artery; MyD88, myeloid differentiation primary response protein; NFkB, nuclear factor kappa B; TLR, toll-like receptor; and VStrep, viridans streptococcus.

for recognizing bacteria were highly upregulated in atherosclerotic plaques in comparison to healthy LITA samples (Figure 6P). Compared with control LITA arteries, the fold rates for TLR2 and TLR 4, for example, were 2.10 and 2.94, respectively. In pathway analysis, both the TLR2/TLR6/CD36 and TLR4/CD14/LY96/LBP metabolic routes were highly significantly upregulated, with the Kyoto Encyclopedia of Genes and Genomes TLR signaling pathway (ID 04640) being the most significantly upregulated (enrichment score=0.721, nominal *P* value=0.002 and false discovery rate=0.046). In this pathway, 57 out of the 97 genes expressed in the atherosclerotic plaques and LITAs (from 102 genes) had at least 1 transcript upregulated (fold change >1.2 and $P<0.05$), and with 54 genes, the most abundant transcript was upregulated in atherosclerotic plaques in comparison to controls.

## DISCUSSION

The role of inflammation in mediating the risk in atherosclerotic cardiovascular disease has been confirmed by the CANTOS (Canakinumab Anti-Inflammatory Thrombosis Outcomes Study) trial, demonstrating for the first time that anti-inflammatory therapy with a monoclonal antibody targeting the IL-1β (interleukin-1β) innate immunity pathway lowered the rate of recurrent

cardiovascular events independently of lipid levels.[62] This suggests that the prevention of inflammation may be as important as reducing plasma levels of cholesterol.

In the present study, we found that the atherosclerotic coronary atheromas of sudden cardiac death victims and atherosclerotic endarterectomy specimens from patients with symptomatic cerebrovascular or peripheral vascular disease harbor DNA from several oral bacteria, suggesting the presence of a microbiome. In normal arteries, bacterial DNA findings were less common. The most common DNA belonged to viridans streptococci—a group of oral bacteria commonly associated with endocarditis and sepsis.[42] Bacterial DNA profiles were similar between autopsy coronary plaques and carotid and peripheral arterial samples obtained under sterile surgical conditions. We confirmed this DNA finding by means of immunohistochemistry, using a pooled in-house antibody raised against the 3 most common viridans streptococcal species detected in atheromatous plaques. The occurrence of viridans streptococcal DNA in coronary plaques correlated with the streptococcal immunopositivity of the same samples, suggesting that this is not a matter of cross-reactivity with oxLDL epitopes, although they also may play a role in the initial phase. The most unique finding in our study was that viridans streptococci seemed to colonize the lipid core and wall of an atheroma as a biofilm and that this biofilm was not recognized by cells of the innate immune system. Phenotypically virulent, strongly immunopositive streptococci that were dispersed from the biofilm were seen infiltrating the fibrous cap of the ruptured or symptomatic atheromas. These virulent streptococci were detected by macrophages co-localizing with molecules of the pathogen-recognizing TLR-dependent signaling pathway and with lymphocytes expressing a T-cell receptor complex, indicating the activation of adaptive immunity. GWE analysis of atherosclerotic plaques from endarterectomy patients showed that genes in the TLR-dependent pathways for recognizing and killing bacteria were upregulated. Viridans streptococcal immunopositivity correlated with the severity of atherosclerosis in the clinical and autopsy series, and, in the autopsy series, with death due to CHD or MI. This evidence suggests that viridans streptococci are not innocent bystanders in the plaque.

Most chronic infections are currently considered to be due to bacterial biofilms.[25] The viridans streptococci group is characterized by its unique ability to attach onto various surfaces and initiate the development of a dental biofilm.[38] In the form of a biofilm, bacteria are mainly beyond the reach of antibiotics given in therapeutic concentrations and evade the innate immune system by liberating extracellular polymeric substances that prevent the outer wall structures of the bacteria from being recognized.[63] In contrast, during bacteremia following dental procedures, for example, or in connection with other infections, when the bacteria are in the "planktonic" state in the blood harboring a capsule, they can be eliminated by orally administered antibiotics.[39]

In the ruptured coronary atheromas of fatal MI cases in our series, the viridans streptococcal antibody cocktail stained intensely discrete groups of bacteria-like structures colocalizing with CD68-positive macrophages not far from the biofilm. These bacteria were also detected in masses outside the macrophages and were observed to have invaded the fibrous caps of atheromas in the ruptured and thrombosed coronary atheromas of MI cases, as well as in the symptomatic carotid arteries of clinical endarterectomy patients. This finding is consistent with the knowledge of the life of bacterial biofilms, inside which the next generation of virulent phenotype bacteria (eg, *Streptococcus pneumoniae*) builds up and is dispersed due to some as yet poorly known triggering factors, such as respiratory viruses, norepinephrine, limited nutrition,[57] a fatty acid messenger, or free iron.[64] This might also offer an explanation for the increase in the risk of MI in association with influenza, pneumonia, acute bronchitis, and other chest infections.[65]

In a normal artery, the entry of bacteria is prevented by the innate immune system, including the opsonization of the bacteria by complements and phagocytosis by macrophages.[2] The development of hypoxia inside a growing atheroma signals the formation of new neovascularization channels from the luminal side and adventitia,[60] opening up a direct gateway for bacteria to enter the atheroma during transient bacteremia. In concordance with this, in our study, we often found neovascular vessels not far from the bacterial biofilm.

Bacteremia is common in the course of acute infections, such as pneumonia, a urinary infection, skin infections, or enteric infections. Bacteremia is also a known consequence of dental procedures, such as root canal treatment, tooth extraction, the treatment of periodontitis, and the removal of dental plaque.[39] Previously, we have found that sudden cardiac death victims had suffered from poor dental health.[66] Among clinical patients with MI, we have found a significant association between the presence of periapical abscesses and oral viridans streptococci DNA–positive thrombi aspirates.[28]

In our experimental setting, all 3 gram-positive streptococci species and the gram-negative bacterial strains mainly activated the TLR2 receptor at higher concentrations. However, in some ruptured atheromas, we found an expression of TLR4 along with the expression of TLR2. It has been reported that, during the growth phase, gram-positive *Streptococcus pneumoniae* release

pneumolysin that interacts with TLR4.[67] In addition to viridans streptococci, which function as early invaders, a coronary plaque biofilm may harbor other oral or even gut bacteria,[68] including other gram-negative bacteria besides *Porphyromonas gingivalis* or *Aggregatibacter actinomycetemcomitans*, such as *Veillonella*[33] or *Prevotella*.[38] These bacteria may prefer TLR4. DNA of other oral bacteria were detected in 5% to 20% and that of *Chlamydia pneumoniae* in 3.8% to 10.5% of the samples, suggesting a possible role in the colonization of the biofilm. In our series, bacteria-like particles inside macrophages that stained positively with the viridans streptococcal antibody cocktail also stained positively with macrophage marker CD68, MyD88, and NF-kB, along with macrophage membrane–bound TLR2 and TLR4 and their coreceptor CD14.[12] This suggests that the TLR-dependent bacteria-recognizing pathway has been activated and elicited downstream signaling via the NF-κB pathway to destroy the bacteria. This is also supported by our GWE findings on the activity of signaling pathways in clinical endarterectomy specimens. Moreover, in the same location in the coronary atheroma, there was already an intense staining with CD 247 and CD3, which form a T-cell receptor complex at the surface of T cells, indicating the activation of an adaptive immune response.

Preimmune serum did not stain streptococcal biofilms (see Figures 2 and 4) but did stain free and phagocytized bacteria that were probably dispersed from the biofilm of a ruptured atheroma (see Figure 5). Flow cytometric analysis of the pooled viridans streptococcal antibody (Data S1 and Figure S1) showed that preimmune serum detected *Streptococcus mitis* and *Streptococcus gordonii*, but not *Streptococcus sanguinis*. Nonimmune binding of immunoglobulins to bacteria via the capsular Fc fragment is involved in the opsonization and phagocytosis of bacteria. Most species of streptococci have been found to possess IgG Fc-receptor binding activity.[61] In a biofilm, bacteria have a reduced capsule due to downregulated capsular polysaccharide production,[58] probably explaining the negative staining of the biofilm by preimmune serum.

The key histopathological findings associated with regions of rupture of the fibrous cap of the coronary plaque include a thin fibrous cap, increased signs of inflammatory activity and heightened amounts of proteases that digest fibrous components of the cap.[69] In our study, virulent streptococci, that most probably have been released (dispersed) from the biofilm, were detected by TLRs of phagocytic macrophages initiating an immune response via the MyD88 dependent pattern recognition pathway. The activation of MyD88 dependent pathway leads to the production of cytokines including IL-6 and TNF,[70] which in turn induce the expression of matrix metalloproteinases[71] that degrade collagen causing weakening and rupture of the fibrous cap of the atheroma.[72] This was most probably seen in 1 of our cases, where macrophages loaded with immunopositive viridans streptococci were seen to infiltrate the thin ruptured fibrous cap of a victim who died of fatal MI.

The weakness of our study is that, although a medicolegal autopsy series comprising out-of-hospital deaths is considered the best available sample of the community-dwelling population, it is biased by the overrepresentation of male patients, violent deaths, and alcohol-related deaths.[73] Furthermore, we were able to apply RT-qPCR analysis to only a small proportion of the clinical endarterectomy samples due to the small size of the samples, as most of the samples were used for the GWE study and histology. The calcification of small fragments was also challenging for DNA extraction, and a portion of the RT-qPCR findings may therefore be false negative. That is why we were unable to correlate the qPCR findings with streptococcal immunohistology in the clinical series. Furthermore, most of the endarterectomy samples studied by means of immunohistology were so small that it is again possible that there were false negatives among the samples. Using LITA samples as controls[46] may also be criticized, but ethical considerations prevent the obtaining of healthy peripheral artery samples from living patients. The possibility of postmortem contamination or bacterial contamination during storage of the samples seems unlikely because in that case also, the healthy coronary segments and LITA controls should carry bacteria. Moreover, postmortem contamination does not cause immune response, which was clearly demonstrated in our coronary arteries. The bacterial DNA profile of autopsy coronaries and endarterectomy samples was similar, further supporting our view that postmortem contamination does not explain our results. One of the weaknesses in our study is that it is not possible to distinguish if virulent bacteria were part of the biofilm or arrived newly at the plaque. Both explanations are possible but most probably the virulent bacteria are dispersed from the biofilm and not the other way around because this occurs typically in the life cycle of a biofilm.[57] The strength of our study is that we were able to use human atherosclerotic plaques obtained at autopsy from individuals who had suffered a sudden cardiac death, in addition to obtaining samples from symptomatic clinical patients undergoing vascular procedures. One of the main obstacles in experimental research on MI has been that, in contrast to human disease, atherosclerotic lesions in the commonly used genetically modified mice seldom develop plaque disruption with thrombosis. Moreover, both the anatomy of murine coronary arteries and the murine immune system differ from those of humans. Therefore, parallel studies in human biobanks and clinical studies have been considered important.[1]

## CONCLUSIONS

Overall, the present results suggest that the change from a stable soft-core coronary atheroma into a vulnerable rupture-prone coronary plaque, as well as the development of a symptomatic peripheral artery plaque, may be contributed to by a chronic bacterial infection in the form of a dormant biofilm that colonizes the lipid core and wall of the atheroma and evades immune detection. The biofilm may activate and release virulent-phenotype bacteria capable of invading and rupturing the fibrous cap of the atheromas. This finding adds to the current conception of the pathogenesis of MI and opens new possibilities for the diagnostics and prevention of the fatal complications of atherosclerosis, in addition to focusing on biofilms as targets for new antiatherosclerotic therapies.

## ARTICLE INFORMATION

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### Disclosures

Reijo Laaksonen is affiliated with Zora Biosciences. The remaining authors have no disclosures to report.

### Supplemental Material

Data S1
Figures S1–S4

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